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MECHANICAL RELAXATION RATE AND METABOLISM STUDIED IN FATIGUING MUSCLE BY PHOSPHORUS NUCLEAR MAGNETIC RESONANCE

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SUMMARY

1. We have used phosphorus nuclear magnetic resonance (^{31}P NMR) to study muscular fatigue in anaerobic amphibian muscle. In this paper the biochemical and energetic changes that result from a series of tetani are related to the decrease in rate constant ($1/\tau$) for the final, exponential, phase of relaxation.

2. Using ^{31}P NMR we have measured the concentrations of phosphocreatine (PCr), inorganic phosphate (P_i) and ATP as well as the internal pH. From our measurements we have calculated [creatine], [free ADP], the free-energy change (more precisely, the affinity $A = -dG/d\xi$) for ATP hydrolysis and the rates of lactic acid production and of ATP hydrolysis.

3. We have found that $1/\tau$, the rate constant of relaxation, is correlated with each of the following, independently of the pattern of stimulation: isometric force production, all of the measured or calculated metabolite levels, pH and $dG/d\xi$.

4. There is a clear dependence upon the pattern of stimulation of the relation between $1/\tau$ and each of the following: total duration of the experiment, number of contractions, rate of lactic acid production and rate of ATP hydrolysis.

5. The rate of relaxation is linearly related to [PCr], [creatine], [P_i] and $dG/d\xi$. It is nonlinearly related to isometric force, [ATP], [H^+] and rate of ATP hydrolysis.

6. We conclude that the change in $1/\tau$, like that of isometric force, depends upon metabolic factors, and not upon any *independent* changes in the activation or deactivation of contraction. We suggest that $1/\tau$ may depend upon the free-energy change for ATP hydrolysis which in turn may be related to the rate of Ca^{2+} uptake into the sarcoplasmic reticulum.

INTRODUCTION

When muscle fatigues, the amount of force that can be developed during a contraction decreases and the rates of force development and of relaxation are slowed. We have used phosphorus nuclear magnetic resonance (^{31}P NMR) to relate the mechanical manifestations of fatigue to the simultaneous biochemical and energetic changes. In an earlier paper (Dawson, Gadian & Wilkie, 1978) we showed that in repetitively tetanized anaerobic frog muscle the decline in isometric force development is correlated with changes in the levels of metabolites most closely associated

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with the contractile process, and that force development remains proportional to the rate of ATP hydrolysis as the muscle fatigues. The present paper concerns these same experiments, but we now look at the way in which the decline in the rate constant for the exponential phase of relaxation is related to biochemical and energetic changes.

The slowing of relaxation accompanying fatigue was first observed in isolated muscle over a century ago (see Mosso, 1915) and the phenomenon has been of interest since that time as a means of investigating the nature of contraction itself. It has been postulated that the rate constant for the exponential phase of relaxation is equal to that for crossbridge detachment and thus to the steady rate of crossbridge cycling during the preceding contraction (Edwards, Hill & Jones, 1975*a*, *b*). Alternatively, or additionally, the relaxation rate may be determined by the rate of Ca removal into the sarcoplasmic reticulum (Sandow, 1965; Connolly, Gough & Winegrad, 1971; Briggs, Poland & Solaro, 1977; Blinks, Rüdel & Taylor, 1978) and thus by the mechanism that deactivates crossbridge cycling.

Since the concentrations of various metabolites change considerably during the course of fatigue, it is of interest to ask whether the decline in relaxation rate may be actually *caused* by any of the biochemical changes. In this paper we relate relaxation rate to each of the following: (*a*) isometric force development, (*b*) the concentrations of phosphocreatine (PCr), ATP, free ADP, inorganic phosphate (P_i), H^+ and creatine, (*c*) the affinity, or free-energy change for ATP hydrolysis ($A = -dG/d\xi$) and (*d*) rate of ATP hydrolysis. Of these, only the concentrations of H^+ and of the phosphorus compounds (with the exception of free ADP) can be determined directly by ^{31}P NMR. The other quantities are estimated on the basis of current knowledge of muscle biochemistry, together with the ^{31}P NMR results.

We find that the rate constant for relaxation ($1/\tau$) is indeed a function of the metabolic state of the muscle, regardless of the pattern of stimulation within the limits that we have studied. Unlike force development, $1/\tau$ is not proportional to the rate of ATP hydrolysis, but it is linearly related to the concentrations of some of the products of contraction and to the affinity, or free-energy change for ATP hydrolysis. We make the testable suggestion that $1/\tau$ may depend upon the affinity for ATP hydrolysis which in turn may be related to the rate of Ca^{2+} uptake into the sarcoplasmic reticulum.

METHODS

The techniques that we have developed for studying living muscle by ^{31}P NMR have been described in detail previously (Dawson, Gadian & Wilkie, 1977). The specific methods used in this study, and our reasons for choosing them, are briefly described in our previous paper about these same experiments (Dawson *et al.* 1978).

Physiological methods

The nuclear magnetic resonance sample tube (7·5 mm internal diameter) was converted into an experimental chamber. Pairs of frog (*Rana temporaria*) gastrocnemius muscles were maintained within the spectrometer at 4 °C. These muscles were used because they are large enough (300–400 mg each, wet weight) to yield adequate signal-to-noise ratio in a single experiment. They were made anaerobic in order to achieve a uniform metabolic state throughout the cross-section of the muscle. We found that gassing the Ringer solution with nitrogen is an ineffective means of removing O_2 completely from our system, so 2 mM-NaCN was also added to the Ringer solution 2 h before the commencement of stimulation. Preliminary experiments with an

oxygen electrode indicated that under these conditions, all oxidative metabolism ceases within 45 min of adding NaCN.

The muscles were stimulated (50 Hz sine waves) via two axially placed platinum electrodes and their isometric force development was recorded by a silicon semiconductor strain gauge placed immediately above the sample tube, with output to a chart recorder. Six experiments were done, two using each of the following patterns of stimulation: 1 s every 20 s, 1 s every 60 s, or 5 s every 300 s; the experiments lasted 18–92 min.

Nuclear magnetic resonance methods

^{31}P NMR spectra were recorded at 129·2 MHz on a spectrometer constructed in the Oxford Laboratory and interfaced with a Nicolet B-NC 12 computer as described in detail by Hoult & Richards (1975). The spectrometer was operated in the Fourier transform mode and employed a deuterium field-frequency lock. Spectra were averaged over 2 min (when the muscles were stimulated 1/20 s) or 5 min periods (stimulation at 1/60 s or 5/300 s) and were stored on a magnetic disk. The spectra could then be added to one another as required.

Direct measurements of phosphorus metabolite and hydrogen ion concentration

The concentration of H^+ ions was determined by comparing the position of the P_i peak in the spectra obtained from intact muscles to a pH titration of P_i in a solution of physiological ionic strength at 4 °C. The relative amounts of ATP, PCr and P_i were obtained from measurement of peak areas which were then corrected by the appropriate saturation factors (Dawson *et al.* 1977). The total phosphorus estimated in this way remained constant throughout these experiments; this provides a valuable check on the accuracy of the saturation factors employed.

Although it is possible to calibrate the corrected peak areas directly in terms of mmol kg^{-1} of muscle (Dawson *et al.* 1977), this procedure is too time-consuming to be practical in each experiment. We therefore assumed that the total metabolic phosphorus (that is the total phosphorus present in PCr, ATP, P_i, and sugar phosphates) in solution within the muscle is 32·7 mmol kg^{-1} and assigned concentrations of metabolites on the basis of their relative corrected peak areas. The amount of total metabolic phosphorus was estimated on the basis that resting muscles in oxygen contain 27 mmol kg^{-1} of PCr, negligible sugar phosphates, and have a PCr/ATP = 6·74 and PCr/P_i = 16·02 (see Table 1, Dawson *et al.* 1977). The effect of choosing a different value for [PCr], say 20 mmol kg^{-1}, would be to alter [PCr], [ATP], [ADP] and [P_i] by the factor 20/27 = 0·74 and to make the free-energy change $dG/d\xi$ more positive by $2{\cdot}3\ln(20/27)$, or 0·69 kJ mol^{-1}.

Calculation of metabolite levels

Creatine concentration [Cr] is calculated from the observed changes in PCr on the basis that in fresh resting muscle the ratio [PCr]/([PCr]) + [Cr]) is 0·85 and that the creatine liberated during activity remains within the muscle. [ADP] that is free in solution is calculated from its equilibrium with ATP, PCr, creatine and H^+, catalysed by creatine phosphotransferase (CPT):

$$[ADP] = [ATP] \times [Cr]/K[PCr] \times [H^+]. \quad (1)$$

Eqn. (1) can be written in this form because H^+ is taken up with approximately unit stoichiometry over the pH range from about 6 up to highly alkaline values; K remains reasonably constant at 2×10^9 M^{-1} if $[Mg^{2+}] > 1{\cdot}5$ mM. Our results suggest that in resting muscle [Mg] > 2·5 mmol l^{-1} (Dawson *et al.* 1978). The calculation assumes that the CPT system remains close to equilibrium at all relevant times, and there is good evidence that this is indeed the case (Gilbert, Kretzschmar, Wilkie & Woledge, 1971; see also Dawson, Gadian & Wilkie, 1979*a*).

Calculation of the free-energy change for ATP hydrolysis

The 'free-energy change' for a chemical reaction is a differential quantity that relates the free-energy of the system, G(J) to the degree of advancement of the reaction, ξ (mol); (see, for example, Everett, 1971, p. 131; Wilkie, 1960, p. 280; Wilkie, 1974). It is thus correctly expressed as $-dG/d\xi$, a function to which the name affinity, A, was given by de Donder in 1922; it is a positive quantity for spontaneous processes. The common usage of ΔG to express $-A$ is misleading and should be abandoned (Quantities Units and Symbols, 1975, p. 16).

We have calculated the affinity for ATP hydrolysis using the method devised by Alberty (1972):

$$\text{maximum work per mol} = A = -\mathrm{d}G/\mathrm{d}\xi = -\{\Delta G^{\circ}_{obs} + RT\ln([ADP] \times [P_i]/[ATP])\} \quad (2)$$

where the concentrations in square brackets are those determined analytically and include all ionised and complexed species. The effects of varying pH and $[Mg^{2+}]$ are accommodated in appropriate variations of ΔG°_{obs} which are shown by Alberty in two-dimensional maps at a variety of temperatures. The affinity for the hydrolysis of ATP in these experiments can be determined from the measured metabolite concentrations by putting together eqns. (1) and (2):

$$A = \Delta G^{\circ}_{obs} + RT\ln([Cr] \times [P_i]/K[PCr] \times [H^+]) \quad (3)$$

Calculation of lactic acid production and phosphorus utilization

The quantity of lactic acid (LA) produced was estimated from the pH changes and the buffering capacity of frog muscle as follows:

$$\Delta[LA] = \Delta\left\{\frac{[\text{protein Hist}]\,[H^+]}{K_{hist}+[H^+]} + \frac{[\text{carnosine}]\,[H^+]}{K_{carn}+[H^+]} + \frac{[P_i]\,[H^+]}{K_{Pi}+[H^+]}\right\} \quad (4)$$

$$= \Delta\left\{\frac{36\times10^{-3}[H]^+}{3{\cdot}162\times10^{-7}+[H^+]} + \frac{14\times10^{-3}[H^+]}{5{\cdot}011\times10^{-8}+[H^+]} + \frac{[P_i]\,[H^+]}{1{\cdot}995+10^{-7}+[H^+]}\right\} \quad (5)$$

The derivation and justification for eqns. (4) and (5), as well as our reasons for preferring them to other methods have been explained in detail previously (Dawson *et al.* 1978; Dawson, Gadian & Wilkie, 1979*b*; Wilkie, 1979*a*, *b*). Total phosphorus utilized (P_{util}; the amount of ATP breakdown that would have been required in the absence of all recovery processes) was calculated according to the following equation:

$$\Delta P_{util} = 1{\cdot}5\Delta[LA] - \Delta[PCr] - \Delta[ATP]. \quad (6)$$

There is some doubt as to the amount of ATP resynthesized per mol LA produced (De Furia & Kushmerick, 1977); however, until this question is resolved we think it best to use the commonly accepted stoichiometry, i.e. three phosphorylations per hexose unit of glycogen. To adopt a different value would not substantially alter our conclusions, notably those based on Fig. 5.

Relation of mechanical to biochemical measurements

When muscles were stimulated for 5/300 s, the mechanical record was compared to the spectrum accumulated during the 5 min preceding the contraction. When stimulation was at 1/20 s or 1/60 s the contraction obtained at the middle of the spectral averaging period was used. The force development was measured half way through each contraction to allow for the small linear fading of force as stimulation continued. The rate of relaxation was found by determining the time required for tension to fall from its value at the beginning of the exponential phase to half that value ($t_{\frac{1}{2}}$): this was expressed as a rate constant $1/\tau = 0{\cdot}693/t_{\frac{1}{2}}$. As was shown by Jewell & Wilkie (1960, see p. 37) and later by Edwards and coworkers (1975*a*, Fig. 1), relaxation becomes slower but remains exponential as muscle fatigues.

Statistical methods

In those figures in which means and standard errors are plotted we could choose preset values of neither variable. In order to summarize the results of all six experiments, the values of Y for given values of X were grouped by linear interpolation between the actual data points obtained in each experiment. The variability thus shown arises mainly from variability between experiments rather than from scatter within the individual experiments. Linear regression analyses, the comparison of slopes and the drawing of confidence limits are as described by Brownlee (1957). Tables of the significance of the correlation coefficient normally do not go beyond $P = 0{\cdot}001$. Since many of our correlations are very much more significant than this, we have made use of the equation given by Snedecor & Cochran (1967, p. 184):

$$t = r\sqrt{[(n-2)/(1-r^2)]} \quad (7)$$

degrees of freedom $= n-2$.

P can then be determined by assuming that t is normally distributed if n is greater than about 30. However, when the significance is high, t is a more meaningful measure and in this paper the value of t is usually given.

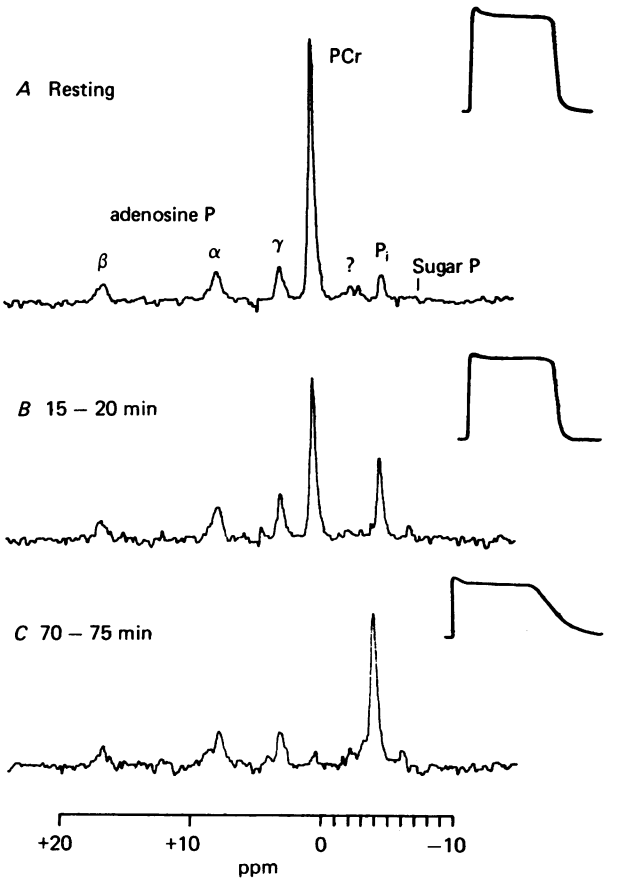

Fig. 1. Spectra and corresponding contractions obtained from two anaerobic gastrocnemius muscles stimulated at 5/300 s. The illustrations of force development have been traced by hand from the original records.

A, resting spectrum obtained after 2 hr anaerobiosis and just before the first stimulation. The peak labelled Sugar P at about −7·5 p.p.m. contains resonances from hexose and triose phosphates. AMP and IMP appear in the same general region. The ? refers to three compounds resonating at −2·7 to −3·6 p.p.m., whose functional significance is unknown. Two of these have been chemically identified, as glycerol phosphorylcholine at −2·7 p.p.m. (Burt, Glonek & Bárány, 1976) and serine ethanolamine phosphodiester at −3·05 p.p.m. (Chalovich, Burt, Cohen, Glonek & Bárány, 1977). Neither the Sugar P nor the ? peaks showed consistent changes in size during the course of stimulation. The insert shows the first contraction of the series, obtained at time zero.

B, spectrum obtained during the period 15–20 min following the commencement of stimulation. The insert shows the fifth contraction in the series, obtained at time $t = 20$ min.

C, spectrum obtained during the period 70–75 min following the commencement of stimulation. The insert is the sixteenth and last contraction, obtained at $t = 75$ min.

RESULTS

Fig. 1 shows a sample of the results from an experiment in which the muscles were stimulated for 5 s every 300 sec. Fig. 1*A* is a resting spectrum taken just before the first stimulation, and shows that the initial [ATP], [PCr], [P_i] and intracellular pH are similar to the values observed in resting aerobic muscle. Comparison of Spectrum 1*A* with Fig. 4 of Dawson *et al.* 1977 shows that when the initial handling

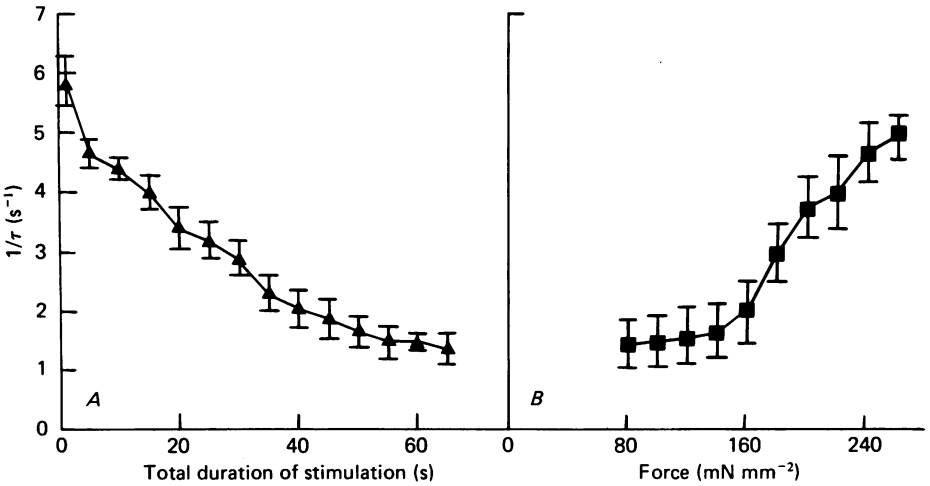

Fig. 2. $1/\tau$ as a function of *A*: total duration of stimulation and *B*: force development. The points represent the combined results of all six experiments. In each experiment the value of *Y* for a given value of *X* was obtained by linear interpolation between the original data points (see Methods). Limits are standard errors.

is followed by 2 h of anaerobiosis (rather than by oxygenation) it results in a slight decrease in PCr and an equal increase in P_i, but no other noticeable changes. The insert to Fig. 1*A* is the first contraction in the series showing the largest force developed (269 mN mm^{-2}) and the fastest relaxation rate ($1/\tau$ = 4·33).

As stimulation continues, the muscles use up their stores of PCr faster than they can be replenished by anaerobic glycolysis; they also show a decrease in pH as a result of lactic acid formation. Spectrum B, obtained after 15–20 min of stimulation, shows a loss of PCr (the PCr peak diminishes in area) a corresponding increase in [P_i] and a shift of the Pi peak upfield (to the left), indicating a change of pH in the acid direction. The insert shows that the contraction obtained at this time develops less force and relaxes more slowly than the initial contraction. The final spectrum and corresponding contraction (Fig. 1*C*) show a continuation of the changes described above. This particular experiment was terminated after 75 min.

The change in relaxation rate constant ($1/\tau$) as fatigue progresses: how this relates to the change in force development

The different patterns of stimulation used in these experiments ensured that there were differences in all of the following: (1) the total duration of the experiment,

(2) the total number of contractions and (3) the total period for which the muscles were stimulated (i.e. the total number of contractions × duration of each stimulation). When $1/\tau$ in individual experiments was plotted as a function of the total duration of the experiment or of the total number of contractions, one of the patterns of stimulation showed a different relationship between these two variables than did the other two. However, when $1/\tau$ was plotted as a function of the total period for

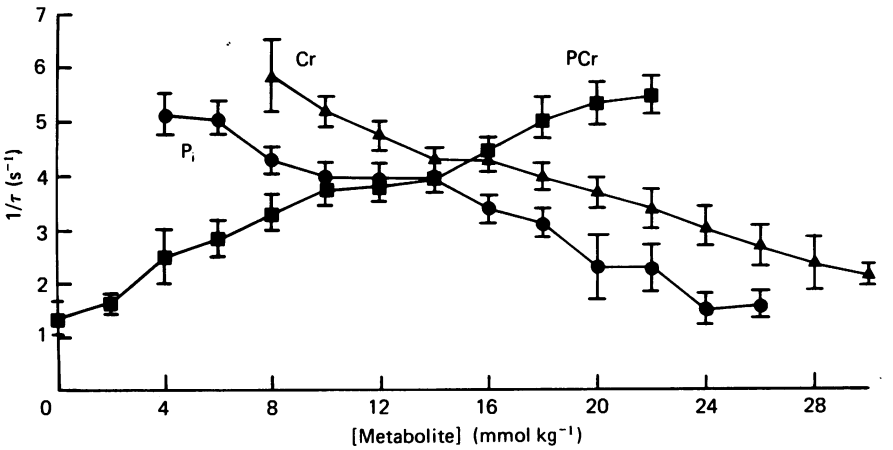

Fig. 3. $1/\tau$ as a function of metabolite concentrations. [PCr] and [P_i] are obtained directly from the spectra and the [Cr] is calculated from [PCr]. Points were interpolated between the original data points (see Methods). Limits are standard errors.

which the muscles were stimulated, the results could not be separated according to the pattern of stimulation. For this reason, the summed results of all six experiments are presented in Fig. 2*A* to show the changes in $1/\tau$ as a function of the total duration of stimulation.

Fig. 2*A* shows that the initial average relaxation rate constant is about 6 s^{-1}, a value that is similar to those obtained previously for brief tetani of frog muscle at low temperature (Jewell & Wilkie, 1960). As the stimulation is continued, $1/\tau$ decreases rather rapidly at first and then more slowly; it may even reach a limit at about 1·5 s^{-1} towards the end of the experiment. Force development declines somewhat differently as stimulation continues, and in Fig. 2*B* $1/\tau$ is compared to the isometric force developed during the same contraction. The relationship between relaxation rate constant and force was independent of the pattern of stimulation, and the results of all six experiments have been averaged after suitable linear interpolation. Fig. 2*B* shows a non-linear relation between relaxation rate constant and force development, with force, unlike $1/\tau$, continuing to decline in extreme fatigue; this change in slope occurs at roughly 160 mN mm^{-2}.

Relaxation rate constant related to metabolite levels

A high degree of correlation, which was independent of the pattern of stimulation, existed between $1/\tau$ and *all* of the metabolite levels that we measured. The average results of all six experiments are shown in Figs. 3, 4*A* and *B* and 5*A*. Fig. 3 shows

that as the muscle fatigues [PCr] decreases to unmeasurably low (approx. < 1 mmol kg^{-1}) values and this change is mirrored by an equal increase in the (measured) [P_i] and the (calculated) [Cr]. Linear regression analysis yielded correlations between each of these metabolite levels and $1/\tau$ that are significant at $P \simeq 0$. (The t value in each case was greater than 15, making P difficult to calculate: e.g. when $t = 6$,

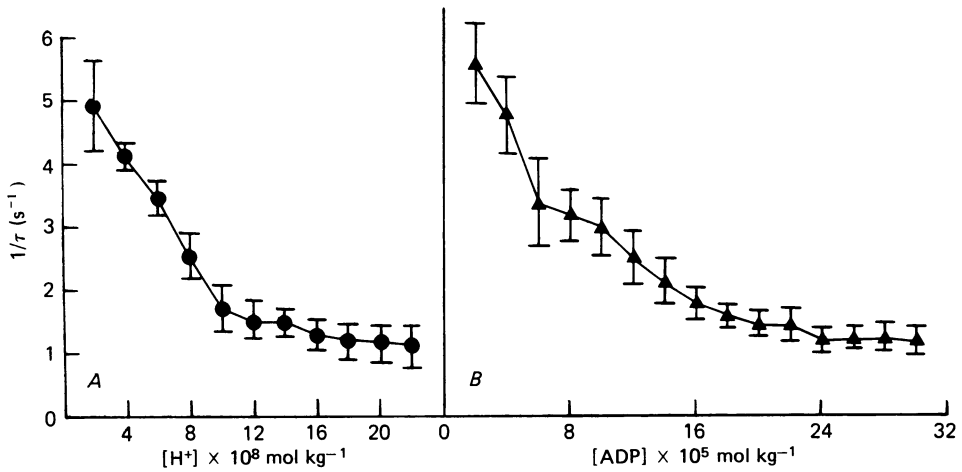

Fig. 4. $1/\tau$ as a function of A: [H^+] and B: free [ADP]. [H^+] is determined from the P_i peak position and [ADP] is determined from the creatine transphosphorylase equilibrium. Points were interpolated between the original data points as indicated in Methods. Limits are standard errors.

$P \simeq 10^{-7}$.) The following equations of best fit were obtained, assuming that all of the experimental error is in the estimates of metabolite concentrations:

$$1/\tau\ (s^{-1}) = 0{\cdot}82 + 0{\cdot}25\ [PCr]\ mmol\ kg^{-1} \quad (r = 0{\cdot}871;\ n = 74;\ t = 15), \tag{8}$$

$$1/\tau\ (s^{-1}) = 7{\cdot}38 - 0{\cdot}25\ [P_i]\ mmol\ kg^{-1} \quad (r = 0{\cdot}877;\ n = 74;\ t = 15{\cdot}5), \tag{9}$$

$$1/\tau\ (s^{-1}) = 8{\cdot}70 - 0{\cdot}25\ [Cr]\ mmol\ kg^{-1} \quad (r = 0{\cdot}871;\ n = 74;\ t = 15). \tag{10}$$

There are also large increases in [H^+] (Fig. 4*A*) and in free [ADP] (Fig. 4*B*). The concentrations of these substances are curvilinearly related to $1/\tau$, with their levels continuing to increase substantially after $1/\tau$ has become virtually constant. The calculated [ADP] that is free in solution in the resting muscles just before the beginning of stimulation was $2{\cdot}89 \times 10^{-5}$ mol kg^{-1} ($\pm 0{\cdot}36$ S.E., $n = 6$). This is roughly 10% of the analytical [ADP] in resting frog muscle, a difference which is accounted for by the binding of ADP to actin and to myosin (Ferenczi, Homsher, Simmons & Trentham, 1978, see their p. 173).

The [H^+] indicated by the resting spectrum taken just before the beginning of stimulation corresponds to a pH of 7·6 ($\pm 0{\cdot}01$ S.E., $n = 6$). This is higher than the values usually quoted for frog muscle (see Bolton & Vaughan-Jones, 1977). However, muscle pH is dependent upon a number of factors, including the p_{CO_2} and the pH of the external medium as well as upon mechanical activity and metabolic state. Tris-buffered Ringer solution was used in these experiments, at a pH of 7·7 (at 4 °C);

the p_{CO_2} was negligible. Due to the high external pH and lack of CO_2 it is very likely that the pH of these muscles was initially greater than would be found *in vivo*.

The changes in [ATP] that occurred during these experiments were small, but significant. The average resting [ATP] just before the beginning of stimulation in all six experiments was 3·4 (± 0·17, S.E.) mmol kg^{-1}, and the average total decrease throughout the experiments was 0·76 (± 0·29, S.E.) mmol kg^{-1}, $P = 0·05$. However,

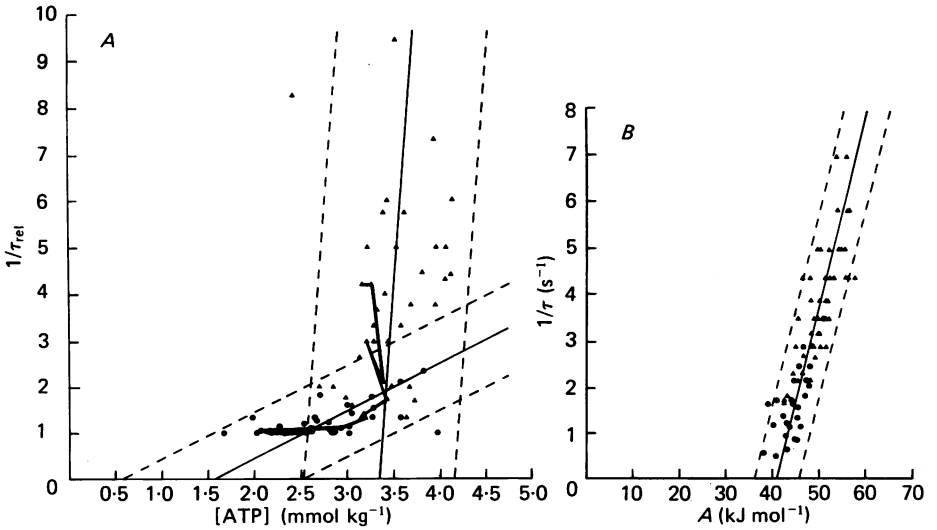

Fig. 5. *A*, $1/\tau_{rel}$ ($1/\tau$ as a multiple of its minimum value) as a function of [ATP]. Data represents results of all six experiments. Each experiment was divided into two parts, initial (represented by ▲) and final, (●). Linear regression analyses were done separately on the two parts, yielding the almost horizontal line for the initial part and the highly sloping line for the second part of the experiment. Both continuous lines are regressions of X upon Y; the dashed lines are 95% confidence limits. The continuous curve represents results of a single experiment in which the muscles were stimulated at 1/60 s. *B*, $1/\tau$ as a function of free-energy change, or affinity, for ATP hydrolysis. Data represent the results of all six experiments. The initial and final parts of the experiments are represented by ▲ and ●, respectively. Continuous line is regression of X upon Y obtained on all the points; dashed lines are 95% confidence limits

almost all the change in [ATP] occurred towards the end of the experiment when the rate constant for relaxation remained fairly constant. This pattern of discontinuity was observed in each individual experiment, as shown by the example in Fig. 5*A* but tended to become obscured when the results were amalgamated, using any method that we have been able to devise. The best way to show this pattern in the combined results was to express $1/\tau$ as a multiple of its minimum value. Each experiment was then divided into two parts, the division being made at the point where the relation between force and relaxation rate constant (see Fig. 2*B*) changed suddenly in slope (the reason for choosing this point of division is explained in 'the metabolic recovery processes' section of Results).

Fig. 5*A* shows the two linear regression lines and 95% confidence limits thus obtained, assuming that all of the experimental error is in [ATP]. No significant

correlation was observed between $1/\tau$ and [ATP] during the first part of the experiment (triangles in Fig. 5*A*, eqn. (11)); this is to be expected when one of the parameters is approximately constant. The correlation between [ATP] and $1/\tau$ during the second part of the experiment (circles in Fig. 5*A*, eqn. (12)) was significant at $P < 0{\cdot}001$.

First part of experiment

$$[\text{ATP}]\ \text{mmol kg}^{-1} = 0{\cdot}043\ 1/\tau_{\text{rel}} + 3{\cdot}33 \quad (r = 0{\cdot}203;\ n = 38;\ P > 0{\cdot}1). \quad (11)$$

Second part of experiment

$$1/\tau_{\text{rel}} = -1{\cdot}834 + 1{\cdot}11\ [\text{ATP}]\ \text{mmol kg}^{-1} \quad (r = 0{\cdot}56;\ n = 36;\ t = 3{\cdot}9;\ P < 0{\cdot}001). \quad (12)$$

The difference in slope of the two regression lines was tested (Brownlee, 1957, p. 66) and found to be very highly significant ($P \simeq 0{\cdot}0002$). It is thus clear that the best fit for this data is *not* a single linear relation between $1/\tau$ and [ATP].

Affinity for ATP hydrolysis

The affinity or free-energy change, is a measure of the maximum amount of work theoretically obtainable per mole of ATP hydrolysed (see Methods). Determination of $dG/d\xi$ requires estimates of [ATP], [ADP], [P_i], [Mg^{2+}] and [H^+]; ^{31}P NMR is the only technique that allows all of these quantities to be estimated simultaneously and *in vivo*. Although the changes in $dG/d\xi$ are small (total change approximately 15 kJ mole^{-1}), they do have the same time course as the changes in relaxation rate constant, both occurring mainly during the first half of the experiment. Fig. 5*B* shows a highly linear relationship between these two variables, and linear regression analysis yields the following equation of best fit, assuming that all of the experimental error is in the estimates of affinity.

$$1/\tau\ (\text{s}^{-1}) = -16{\cdot}21 + 0{\cdot}397\ A(\text{kJ mol}^{-1}) \quad (r = 0{\cdot}85;\ n = 74;\ t = 13{\cdot}7,\ P \simeq 0). \quad (13)$$

The metabolic recovery processes

Under anaerobic conditions the resynthesis of ATP occurs mainly from the breakdown of PCr and from glycolysis. Because we are able to estimate the extent of both these recovery processes, we are also able to estimate the amount of phosphorus utilization, P_{utl}, that is the total amount of ATP that would be broken down in the absence of any recovery processes. An interesting result of these experiments is that for a given amount of phosphorus utilization (combining contraction and recovery) a fixed proportion of the ADP is rephosphorylated via PCr hydrolysis and a fixed proportion via lactic acid formation, regardless of the pattern of stimulation. This is shown by Fig. 6, in which both P_{utl} and recovery via PCr hydrolysis are plotted as a function of time for all six experiments. The time axis has been adjusted so that the results of the three patterns of stimulation fall together. The difference between the two curves represents recovery via glycolysis.

Fig. 6 shows that the factor(s) determining the rate of glycolysis under the conditions of our experiments are closely linked to the rate at which phosphorus is being

utilized. Glycolysis proceeds at a constant rate throughout most of each experiment: about 1·35, 0·6 and 0·3 mmol kg^{-1} min^{-1} for 1/20 s, 1/60 s and 5/300 s stimulation, respectively (see Fig. 4, Dawson *et al.* 1978). This rate must be submaximal at least in the cases of the two less demanding patterns of stimulation. It is interesting that as early as 1928 Eggleton & Eggleton (1928) concluded that under anaerobic conditions the PCr hydrolysis mainly precedes glycolysis in both resting

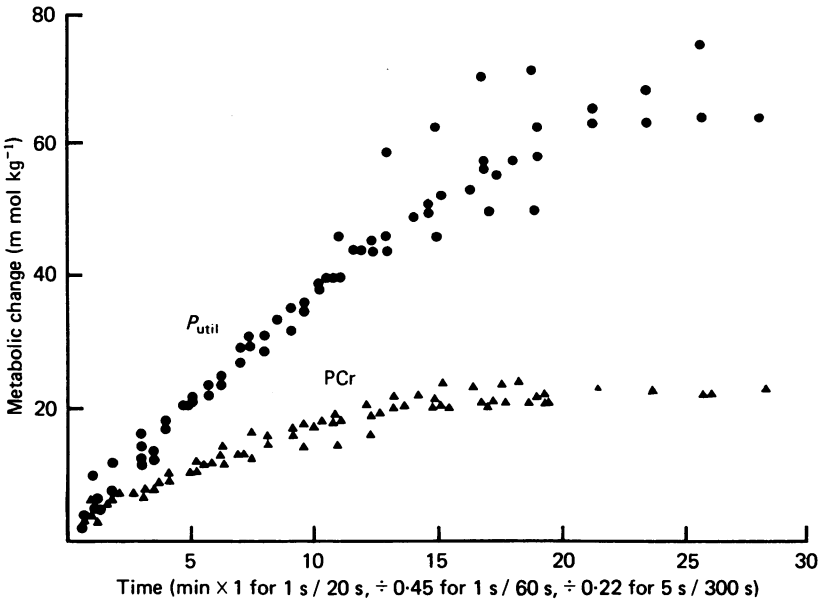

Fig. 6. Total phosphorus utilization (●) and total PCr hydrolysis (▲) as a function of time in all six experiments. The x-axis has been adjusted to make the results of all six experiments coincide. One unit on the time scale denotes 1 min when the muscles were stimulated 1/20 s, 2·2 min when stimulated 1 s/min and 4·5 min when stimulated 5 s/5 min. The difference between the two plotted curves represents recovery via glycolysis.

and contracting muscle; this was revolutionary at the time, since it was generally believed that lactic acid itself was the direct cause of contraction. From the viewpoint of animal design it is surprising that glycolysis is not turned on more fully by the milder patterns of stimulation, for this would seem to provide a better stratagem for conserving PCr to use in emergencies.

The amount of PCr available to contribute to recovery eventually becomes negligible. This change from two sources to a single source of rephosphorylation of ADP occurs when force development has fallen to about 160 mN mm^{-2}. This is the same point at which the relation between force development and relaxation rate constant ceases to be linear (Fig. 2*B*) and is the point at which *all* the curvilinear relations between biochemical quantities and either force development or relaxation rate constant show their major curvature.

The relation between $1/\tau$ and rate of ATP hydrolysis

Fig. 7 shows the relation between $1/\tau$ (expressed as a multiple of its minimum value and designated $1/\tau_{rel}$) and the rate of ATP hydrolysis per second of stimulation $\dot{P}_{util}$ mmol kg^{-1} s^{-1}) in each of the four experiments in which the muscles underwent repeated 1 sec contractions. These results show a highly discontinuous relationship: $1/\tau_{rel}$ falls steeply with slight, if any, reduction of $\dot{P}_{util}$, then remains constant while $\dot{P}_{util}$ falls to a very low level.

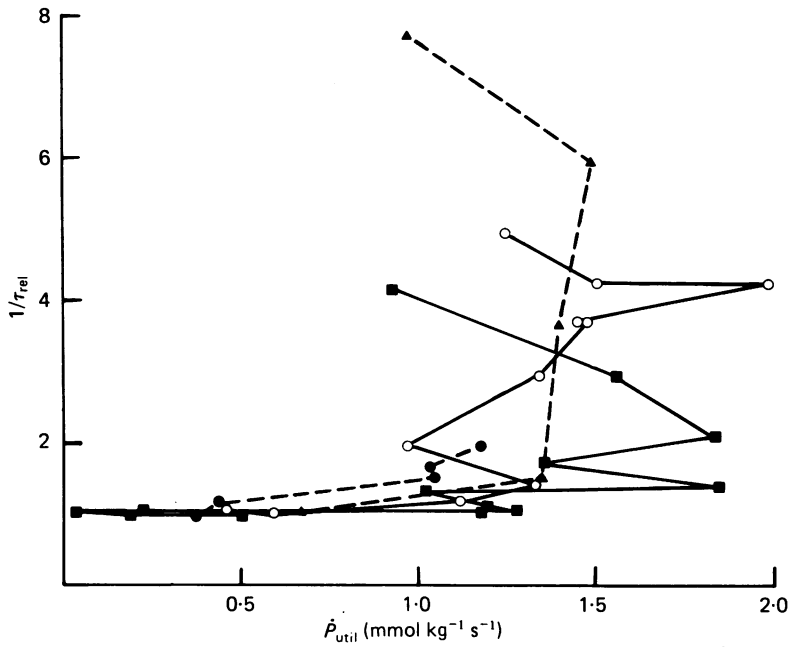

Fig. 7. $1/\tau_{rel}$ ($1/\tau$ as a multiple of its minimum value) as a function of $\dot{P}_{util}$ in the four experiments using 1 s contractions. Continuous lines indicate stimulation at 1/60 s and dashed lines indicate stimulation at 1/20 s.

Because of the large scatter in the data shown in Fig. 7, we felt it necessary to test statistically whether these results are indeed fit better by two linear equations than by a single one. To do this the experiments were divided into first and second parts as described for Fig. 5*A*, and linear regression analyses were done separately on the two parts. The equation for X upon Y (assuming all of the experimental error is in $\dot{P}_{util}$) thus obtained for the first part of the experiment was highly significantly different from both those (X upon Y and Y upon X) obtained for the second part (t = 4·3 and 11 respectively). We therefore conclude that our results are not consistent with the hypothesis that $1/\tau$ is proportional to $\dot{P}_{util}$.

DISCUSSION

Skeletal muscle shows a characteristic pattern of relaxation following a tetanic contraction. After the last stimulus there is a delay, during which isometric tension falls only slightly, followed by an exponential return to the resting level. While the time to onset of the exponential phase can be altered simply by changing the mechanical recording conditions (Huxley & Simmons, 1972), the *rate constant* of the exponential phase is independent of such changes.

Jewell & Wilkie (1960) showed that the rate constant for relaxation is the same following isometric or isotonic contractions and that it is independent of the load that the muscle bears during the contraction. They also showed that $1/\tau$ is not affected by substantial length changes during which several crossbridges would be broken and remade nor by insertion of added compliance into the measuring system in series with the muscle. Such effects might arise, though to a lesser degree, if one part of the muscle relaxed earlier than another. They concluded that the exponential phase of relaxation does not arise from the characteristics of passive elements in the muscle, but reflects the progress of some underlying biochemical reaction(s). It has been shown that internal length changes do indeed occur during the exponential phase of relaxation (Huxley & Simmons, 1970; Edman & Flitney, 1977); these are smaller in extent than the experimental changes imposed by Jewell & Wilkie, and thus neither alter their conclusion nor affect the reasoning behind the present paper.

Our present results demonstrate that the mechanical manifestations of fatigue are indeed related to the progressive metabolic changes in fatiguing muscle. We use three different patterns of stimulation in order to vary the rate at which fatigue develops and find that the results all fall together, independently of the pattern of stimulation. This is the case when in the individual experiments either force development (see Dawson *et al.* 1978) or $1/\tau$ are plotted as a function of *any* of the metabolite levels most closely associated with contraction or as a function of the affinity or free-energy change for ATP hydrolysis; this is in marked contrast to the fact that when the mechanical variables are plotted as a function of time or of the number of contractions the results are clearly separable on the basis of the pattern of stimulation.

In our previous paper on these same experiments we reported that the results of the experiments using 5 s contractions are separated from those using 1 s contractions if *force development* is plotted as a function of total period of stimulation (number of contractions times the duration of contraction). When $1/\tau$ is plotted as a function of the total period of stimulation no such separation can be made, suggesting that, unlike force, $1/\tau$ could be related either to the metabolic state of the muscle or to the total period of stimulation. The reason for this discrepancy is apparently the somewhat greater scatter in the measurements of $1/\tau$: it is quite clear that $1/\tau$ is correlated with force and with several metabolite levels (e.g. PCr, P_i, Cr) which are demonstrably *not* uniquely related to the total period of stimulation.

We conclude that both the decrease in force development and the decline in relaxation rate are dependent upon the biochemical state of the muscle, although the exact form of the dependence is quite different according to which mechanical variable one is examining. Changes in the activation or deactivation of contraction could only be responsible for the mechanical changes if they are themselves somehow linked to metabolic factors. Such a dependence of mechanical performance upon the

metabolic state of the muscle cannot be surprising in view of the long established fact that muscle fatigues more quickly under anaerobic than under aerobic conditions (e.g. Hill & Kupalov, 1929).

Edwards *et al.* (1975*a*, *b*) also found that mechanical fatigue is related to the biochemical state of the muscle, and were the first group to develop a comprehensive theory relating relaxation rate to [ATP] and rate of ATP splitting during contraction. However, our results differ from theirs in specific ways which will be discussed below.

Relation of relaxation rate constant to metabolite levels

Since we found that $1/\tau$ was related to all of the metabolite levels we measured, independently of the pattern of stimulation, it was not possible to determine which of these relationships is causal. However, some possibilities can be ruled out, and some positive suggestions can be made:

(1) It is unlikely that the rate of relaxation is directly dependent upon the levels of PCr or Cr even though both of these metabolites are linearly related to $1/\tau$ in these experiments. The primary role of PCr and Cr in muscle is to buffer the ATP and ADP levels; thus there are no biochemical grounds for postulating that either the change in [PCr] or in [Cr] is directly responsible for any of the mechanical manifestations of fatigue.

(2) Changes in [ATP] have been postulated to be responsible for the prolongation of relaxation (Edwards, Hill & Jones, 1972, 1975*a*) and in mouse soleus muscle the change in [ATP] appeared to be linearly related to the change in half-time for the exponential phase of relaxation as fatigue progressed (Edwards *et al.* 1975*a*). We find a very different pattern in our experiments (see Fig. 5*A*): During the first part of the experiment, over which $1/\tau$ changes by about threefold, there is virtually no change in [ATP], while the small changes in [ATP] that do occur are towards the end of the experiment when $1/\tau$ remains fairly constant. In the experiments of Edwards *et al.* (1975*a*) on mouse soleus muscle the changes in [ATP] were also small (about 1 mmol kg^{-1} wet weight), and it is possible to detect a similar discontinuity in the relation between $1/\tau$ and [ATP]. Replotting the data shown in Figs. 3 and 4 of their paper as [ATP] against $1/\tau$ yields a result that is very similar to our Fig. 5*A*. We conclude that the rate constant for relaxation is not determined by the [ATP].

(3) A total of almost 1 mmol kg^{-1} ATP is broken down during our experiments, but the increase in free ADP is only about 30 μmol kg^{-1}. The ATP that is lost is presumably converted first to AMP, then to IMP via the myokinase and adenosine monophosphate deaminase reactions. Both AMP and IMP appear in the spectrum at $-6{\cdot}6$ p.p.m., and therefore cannot be distinguished from the sugar phosphate (see legend to Fig. 1). In any case, their concentrations are too low, even at the end of the experiments to be measured by NMR. However, since neither AMP nor IMP is known to be involved in any of the processes causing a transition from the active to the resting state, it is unlikely that changes in concentration of either of these metabolites could be directly responsible for the decrease in $1/\tau$.

(4) It has in the past been postulated that lactic acid formation and the consequent increase in $[H^+]$ is responsible for the decreased relaxation rate that accompanies fatigue. This hypothesis was based on the finding that interventions expected to reduce intracellular pH cause a decline in relaxation rate in a variety of muscles. It

was long ago discovered that acid solution decreases relaxation rate in cardiac muscle (Gaskell, 1880) and that exposure to high [CO_2] decreases relaxation rate in frog gastrocnemius muscle (Waller & Sowton, 1896) and in snail retractor pharynx, a smooth muscle (Bozler, 1930). However, prevention of the usual increase in acidity by poisoning with iodoacetic acid does not prevent the decline in relaxation rate in repetitively stimulated amphibian muscle (Parkinson, 1933) or in fatigued mouse soleus muscle (Edwards *et al.* 1975*a*). Taken together, these results suggest that relaxation rate in fatiguing muscle must be affected by, but is not solely determined by, changes in [H^+].

(5) A dependence of relaxation rate upon the combined products of ATP hydrolysis (H^+, ADP and P_i) could make sense no matter whether relaxation rate is determined by crossbridge cycling rate or by the rate of Ca^{2+} removal from the sarcoplasm, or both.

(*a*) It has been shown that in myosin solution the later stages of ATP hydrolysis are reversed by high concentrations of product (Trentham, Eccleston & Bagshaw, 1976). If a similar effect occurs *in vivo*, changes in product concentration could affect crossbridge cycling, and therefore force development (see Dawson *et al.* 1978) and/or relaxation rate.

(*b*) Uptake of Ca^{2+} into isolated vesicles of sarcoplasmic reticulum is also known to be dependent upon the products of ATP hydrolysis: Ca uptake by isolated sarcoplasmic reticulum is inhibited by high [ADP] (Hasselbach & Makinose, 1962) and is pH dependent (see Hasselbach, 1974, p. 447). Ca previously stored in vesicles of the sarcoplasmic reticulum is released and a net synthesis of ATP can be demonstrated when ADP and P_i are added to incubation medium containing little ATP (Barlogie, Hasselbach & Makinose, 1971; Panet & Selinger, 1972) suggesting strongly that the calcium pump is actually reversible. There is insufficient information to relate these results obtained *in vitro* quantitatively to the situation that we observe *in vivo* so we cannot say with assurance that the combined changes in product concentration that occurred in our experiments are of sufficient magnitude to affect the uptake of Ca^{2+} into the sarcoplasmic reticulum: nevertheless this seems to be very likely as explained in the following section.

Relation of relaxation rate constant to affinity for ATP hydrolysis

The affinity for ATP hydrolysis decreases as stimulation continues, mainly as a result of increases in the product concentration, rather than a decrease in [ATP] (see eqn. (3)). Since the correlation between $1/\tau$ and the calculated affinity for ATP hydrolysis is extremely high, and the relationship is a simple linear one (see Fig. 5*B*), it is worth considering how changes in affinity could cause changes in $1/\tau$. Both crossbridge cycling and Ca^{2+} uptake into the sacroplasmic reticulum are ATP-dependent, energy-requiring processes, so either one or both of these might be affected when the affinity for ATP hydrolysis is decreased. The present studies yielded no indication that the decline in affinity for ATP hydrolysis is related in any simple fashion to the decrease in force development as the muscle fatigues (contrast Fig. 3*b* of Dawson *et al.* 1978 with Fig. 5*B* of the present paper). On the other hand, a simple relation between affinity for ATP hydrolysis and rate of Ca^{2+} uptake into the sarcoplasmic reticulum does suggest itself.

Ca is taken up into the sarcoplasmic reticulum against its concentration gradient by an ATP-dependent transport system. The capacity of the reticulum to sequester Ca is greatly extended by the presence of internal Ca binding proteins (see MacLennan & Holland, 1975), and it is generally believed that the bound and free calcium of the reticulum are in equilibrium with each other (Endo, 1977, p. 75; MacLennan & Holland, 1975, p. 393). The total affinity available to drive the uptake of Ca^{2+} into the sarcoplasmic reticulum, $-(dG/d\xi)_{total}$, is

$$-(dG/d\xi)_{total} = -(dG/d\xi)_{ATP} - n(dG/d\xi)_{osm}, \quad (14)$$

where $(dG/d\xi)_{ATP}$ is the free energy change per mol ATP hydrolysed (a negative quantity) and $(dG/d\xi)_{osm}$ (a positive quantity) is the minimum work required to pump a mole of free Ca^{2+} against the concentration gradient, assuming the absence of an electrical potential; n is the number of Ca^{2+} ions taken up per mol of ATP hydrolysed, and is thought to be 2 (Hasselbach, 1974). In the absence of an electrical potential across the sarcoplasmic reticulum membrane, and at 4 °C

$$(dG/d\xi)_{osm} = 5{\cdot}3 \log_{10}\{[\text{free } Ca^{2+}]_{in}/[\text{free } Ca^{2+}]_{out}\} \text{ kJ mol}^{-1}. \quad (15)$$

Combining eqns. (14) and (15) it can be seen that relatively small changes in $(dG/d\xi)_{ATP}$ will have very large effects on the maximum concentration ratio achievable; this feature of all active transport systems seems not to be widely appreciated but may be very important in relation to our Fig. 5*B*.

When approaching equilibrium the affinity ($-(dG/d\xi)_{ATP}$) is only slightly more positive than $n(dG/d\xi)_{osm}$. It has been suggested (Hasselbach, 1974) that it is the decline of $dG/d\xi_{total}$ to zero that actually halts pumping of Ca^{2+} by isolated sarcoplasmic reticulum. Even at some distance from equilibrium, the decrease in affinity for ATP hydrolysis that we observe in fatiguing muscle (Fig. 5*B*) must diminish the rate of sequestration of sarcoplasmic Ca^{2+} during relaxation if this process is indeed rate-limited by an active Ca^{2+} pump. It is impossible at the moment to predict quantitatively the expected relation between affinity for ATP hydrolysis and rate of Ca^{2+} sequestration, because so much remains unsure about the circulation of Ca^{2+} during activation, relaxation and recovery. The most important uncertainties concern the possible existence of an electrical potential across the sarcoplasmic reticulum membrane; the evident topological complexity of the reticulum itself with the likelihood of compartmentation, and the extent and significance of Ca^{2+} binding to proteins both inside and outside the sarcoplasmic reticulum. We have sought but not found, a description of the exact way in which the 3·1 mmol kg^{-1} of total Ca^{2+} (Dubuisson, 1942) is distributed among compartments and complexes, though according to Endo (1977, p. 73) it is within the capacity of the sarcoplasmic reticulum to contain it all. Certainly very little of it is in solution in the sarcoplasm during activity and even less ($\leqslant 10^{-7}$ M) during rest.

Nevertheless, eqns. (14) and (15) agree remarkably well with experimental results. According to Endo (1977) the concentration of free Ca^{2+} inside the sarcoplasmic reticulum may be about 0·5 mM. In order to lower the sarcoplasmic $[Ca^{2+}]$ to 10^{-7} M and thus switch off contraction, the calculated affinity for ATP splitting must be at least 39 kJ mol^{-1}. This is remarkably close to the value (41 kJ mol^{-1}) obtained by extrapolating the line in Fig. 5*B* to the base line, where relaxation would fail completely. All this is compatible with the suggestion that the Ca^{2+} pump is slowed, and finally stopped, by the fall in the affinity for ATP hydrolysis.

At least two other lines of research on living muscle confirm that the removal of Ca^{2+} from the sarcoplasm is indeed slowed as a result of fatigue. Recently, very solid evidence has been provided by Blinks and co-workers (1978) that the rate of relaxation from contraction is related to the *in vivo* rate of Ca^{2+} sequestration (Blinks *et al.* 1978). They found that the progressive decrease in relaxation rate constant in repetitively stimulated muscle is accompanied by a similar slowing of the decay of aequorin response, which they conclude probably results from

slowing of the rate of Ca^{2+} uptake into the sarcoplasmic reticulum. Clearly, this is completely compatible with the suggestion we have made above on energetic grounds.

It is also of interest to consider how our results relate to the Ca^{2+} fluxes in contracting muscle, as described by Winegrad (1968, 1970). Winegrad's autoradiographic studies showed that the fading of force maintained during single long contractions is associated with decreased $[Ca^{2+}]$ in the area of the myofibrils (Winegrad, 1970), and he and his co-workers have found that at two different temperatures the time course of changes in force development and relaxation rate in test twitches immediately following a tetanus are related to the rate of internal translocation of Ca^{2+} within the sarcoplasmic reticulum (Connolly *et al.* 1971). These results are interpreted by the authors to suggest that Ca^{2+} distribution within the SR influences the mechanical characteristics of contraction. If such a redistribution of Ca^{2+} occurs in fatiguing muscle, it could be responsible for both the decline in force development and in $1/\tau$ that are observed in these experiments. The point could be tested either by autoradiographic studies similar to Winegrad's or by use of *in vivo* Ca^{2+} indicators.

Turning to *in vitro* evidence, studies concerning the rate of Ca^{2+} uptake by isolated vesicles of the sarcoplasmic reticulum are consistent with the possibility that the rate is related to the driving force for Ca^{2+} uptake. Ogawa has shown that the rate of Ca^{2+} uptake may decrease linearly with increasing free $[Ca^{2+}]$ within the sarcoplasmic reticulum (Ogawa, 1970) and that when the concentration of ATP and other substrates used to support Ca^{2+} uptake are varied, the rate of calcium uptake is linearly related to the external $[Ca^{2+}]$ required to fill the isolated vesicles to half-maximal capacity (Ogawa, 1972). In each of these cases interventions which must alter the driving energy for Ca^{2+} uptake also altered the rate of Ca^{2+} uptake, suggesting a simple causal relationship.

Relaxation rate constant and rate of ATP hydrolysis

The theory of Edwards *et al.* (1975*a*) is an interesting attempt to relate measurement of $\dot{P}_{\mathrm{util}}$ during contraction (single submaximal tetani of mouse soleus muscle at 24–25 °C; duration 2–60 s) with the rate of exponential tension fall during the subsequent relaxation. They link the two phenomena by a kinetic scheme which concentrates on the final step by which the crossbridges that are at the end of their cycle (X) finally dissociate back into actin (A) and myosin (M).

$$\mathrm{ATP} + \mathrm{AM} \dashrightarrow \mathrm{X} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{A} + \mathrm{M} + \mathrm{ADP} + \mathrm{P_i}. \tag{16}$$

If the production of X (interrupted arrow) is halted abruptly by lowering $[Ca^{2+}]$, the concentration of X declines exponentially with rate constant k_1. A similar change might be expected in the force, since the crossbridges generate and bear it.

When, during contraction, process (16) is in a steady state, and if $\dot{P}_{\mathrm{util}}$ is assumed to equal the rate of ATP splitting by the crossbridges, we have

$$\dot{P}_{\mathrm{util}} = k_1[\mathrm{X}], \tag{17}$$

so if $[X]$ were known, k_1 could be determined, and vice versa. Edwards and co-workers maintained force, and therefore presumably [X], constant as the muscle fatigued by using submaximal stimulation. They found that $\dot{P}_{\mathrm{util}}$ was proportional to the half-time for relaxation, within experimental error.

Unfortunately, all that can be said with certainty about [X] is that it must be less than the analytical concentration of the enzymatically active part of myosin subfragment S1, (the concentration of S1 is thought generally to be about 0·28 mmol/kg wet weight, although as far as we know it has never been determined on frog muscle). Considering the extreme case, where all the S1 is in the X form, k_1 comes out as $0{\cdot}428/0{\cdot}28 = 1{\cdot}5\ \mathrm{s}^{-1}$ for non-fatigued muscle ($\dot{P}_{\mathrm{util}} = 0{\cdot}428$ has been taken from Edwards *et al.* 1975*a*, table 1, 0–15 sec). This is very much slower than the observed rate constant for mechanical relaxation which was about $18\ \mathrm{s}^{-1}$. Even after the authors had applied substantial, though defensible, corrections both to $\dot{P}_{\mathrm{util}}$ and to [X], the calculated breaking of crossbridges was always 2–4 times *slower* than the observed fall of force. A discrepancy in this direction is very hard to explain.

In contrast, when a similar analysis is applied to our own results on repeated tetani of frog gastrocnemii at 4 °C, the calculated rate of breaking of crossbridges is always *faster* than the observed rate of fall of force, by at least 13% in the fresh muscle rising to above 95% as the

muscle becomes fatigued. A discrepancy in this direction can easily be explained. For example, it would arise if the rate of reduction of cytoplasmic [Ca^{2+}] is one of the limiting factors during relaxation.

A similar discrepancy would arise if the very large increases in product concentration shown in Figs. 3 and 4 slowed down the breakdown of X (dotted arrow, k_{-1}, in eqn. (16)). This possibility can, and should, be tested by experiment on actomyosin systems *in vitro*. Probably the most direct evidence that $1/\tau$ is *not* directly proportional to $\dot{P}_{\text{util}}$ is given by our Fig. 7 which shows the highly discontinuous nature of the relation between these two variables. At first $1/\tau$ diminishes rapidly with only slight diminution in $\dot{P}_{\text{util}}$, then $\dot{P}_{\text{util}}$ diminishes markedly with only slight reduction of $1/\tau$. It could be argued that [X] must vary with force in our experiments. However, no simple correction of [X] for changes in force development leads to the relation between $1/\tau$ and $\dot{P}_{\text{util}}$ predicted by eqns. (16) and (17).

The theory of Edwards and coworkers also predicts that the economy of isometric contraction (force/$\dot{P}_{\text{util}}$ or force/rate of heat production) must increase as $1/\tau$ increases, and they have found evidence for increased economy of contraction in fatigued muscles when comparing force development to heat production in submaximally stimulated human quadriceps muscles (Edwards *et al.* 1975*b*). We, however, find in our experiments that force/$\dot{P}_{\text{util}}$ remains unchanged as stimulation is continued. (Dawson *et al.* 1978.) In much earlier studies Feng (1931) found that the relation between heat production and force is constant as frog muscle fatigues for the first several *minutes* of continuous stimulation at 0 °C; he observed an increase in force/heat production only after the force had fallen to about 10 % of its initial value. The available information on frog muscle is thus not compatible with the hypothesis of Edwards and co-workers.

Simplicity is one of the main virtues that one seeks in a theory and it is to be regretted that it would be essential to elaborate the theory of Edwards *et al.* (1975*a*) to accommodate it to all the experimental results. Much more research is needed before devising such a new theory. For example, we do not know how much of the ATP hydrolysis during contraction is actually associated with crossbridge cycling. Nor do we know how the force during relaxation is related to the number of crossbridges, though the time course of return of myosin heads toward the thick filaments following contraction has been studied using time-resolved X-ray spectroscopy and found to be complex (see Yagi, Ito, Nakajima, Izumi & Matsubara, 1977).

Conclusions

We conclude that in fatiguing muscle the decline in rate of the exponential phase of relaxation is, like the decrease in force development, dependent upon the metabolic state of the muscle. This finding confirms by an entirely different method the conclusion of Jewell & Wilkie (1960) that $1/\tau$ reflects the progress of some underlying biochemical reaction(s). We also find that unlike force development, $1/\tau$ is linearly related to the affinity for ATP hydrolysis. Since both crossbridge cycling and Ca^{2+} uptake into the SR are ATP-dependent, energy-requiring processes, they must both ultimately depend upon the affinity for ATP hydrolysis. However, the evidence now available does not allow us to predict precisely how the change observed in these experiments of 15–20 kJ mol^{-1} ATP hydrolysed should affect the rate of either of these processes.

We can suggest no simple mechanism to account for our results on the basis that relaxation rate is related to the rate of crossbridge cycling during the previous contraction. On the other hand, there is strong evidence that $1/\tau$ is related to the rate of Ca^{2+} sequestration in repetitively stimulated muscle (particularly that of Blinks *et al.* 1978) and the relation we observe between $1/\tau$ and affinity for ATP hydrolysis can be simply explained on this basis. We suggest that $1/\tau$ *may* be related to the rate of Ca^{2+} uptake into the sarcoplasmic reticulum during the exponential phase of relaxation, which may in turn depend upon the free-energy change for ATP hydro-

lysis. This suggestion has the valuable attribute that it can be tested by a number of different experimental techniques: if it is incorrect it should be short-lived.

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